TRANSCRIPTION OF HISTONES H1 AND H2B IS REGULATED BY SEVERAL IMMUNE STIMULI IN GILTHEAD SEABREAM AND EUROPEAN SEA BASS

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Abstract

Histones (H1 to H4) are the primary proteins which mediate the folding of DNA into chromatin; however, and in addition to this function, histones have been also related to antimicrobial peptides (AMPs) activity in vertebrates, in fact, mammalian H1 is mobilized as part as the anti-viral immune response. In fish, histones with AMP activity have been isolated and characterized mainly from skin and gonads. One of most threatening pathogens for wild and cultured fish species nowadays is nodavirus (NNV), which target tissues are the brain and retina, but it is also able to colonize the gonad and display vertical transmission. Taking all this into account we have identified the *h1* and *h2b* coding sequences in European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) fish species and studied their pattern of expression under naïve conditions and NNV *in vivo* infection. The data obtained prompted us to study their role on the immune response of gonad and head-kidney leucocytes upon viral (NNV), bacteria (*Vibrio anguillarum* or *Photobacterium damselae*), pathogen-associated molecular patterns (PAMPs) or mitogens stimulation. The *h1* and *h2b* genes are expressed in a wide range of tissues and their expression is modify by infection or other immune stimuli, but further studies will be needed to determine the significance of these changes. These results suggest that *h1* expression is related to the immune response against NNV in the brain, while *h2b* transcription seems to be more important in the head-kidney. Moreover, the potential role of histones as anti-viral agents is suggested and further characterization is in progress.

Keywords: Histones; antimicrobial peptides (AMPs); nodavirus (NNV); head-kidney; brain; gonad; European sea bass; gilthead seabream; teleost

1. Introduction

Histones are usually classified as core (H2A, H2B, H3 and H4) and linker histones (H1) due to their localization forming the basic units of the chromatin, the nucleosome. Thus, the nucleosome is formed by 146 base pairs of DNA wrapped around a protein octamer of two molecules of core histones. The linker H1 binds the DNA at the union sites whether it enters or exits the core nucleosome [1]. Histones, mainly core ones, are greatly conserved in eukaryotic organisms along evolution and therefore their functions might be also conserved. In addition to this function, they are also involved in other cellular functions and their implication in the epigenetic control of gene expression is nowadays in fashion. However, they have been also linked to immunity being their role as antimicrobial peptides (AMPs) the most described, which were first characterized in mammals long time ago [2]. Thus, histones and histone-derived fragments act as physiological barriers of cells exerting a variety of antimicrobial actions and functions, including bacterial cell membrane permeabilization, penetration into the membrane followed by binding to bacterial DNA and/or RNA, binding to bacterial lipopolysaccharide (LPS) in the membrane, neutralizing the toxicity of bacterial LPS, and entrapping pathogens as a component of neutrophil extracellular traps (NETs) [3].

In fish, the connections between histones and immunity have been established. First characterized was a catfish (*Ictalurus punctatus*) AMP isolated from the skin closely related to the H2B [4]. Since then, proteins highly homologous to histones or fragments derived by cleavage processes from histones (eg. Parasin I, hipposin) have been defined as histone-like proteins (HLPs) and identified in some fish species [5-9]. Most studies in fish have focused on the antimicrobial function of HLP-1 and HLP-2 proteins homologous to H2B and H1, respectively; and usually isolated from skin or gills [4, 10-14]. However, other AMPs have been widely distributed among several tissues including immune-privileged tissues such as brain or gonads [15]. Recently, a H1-like protein has been isolated from acidified testis extracts (fH1LP) of olive flounder (*Paralichthys olivaceus*) and shown to be constitutively expressed in ovary and testis and to have antibacterial (Gram+ and Gram-) and antifungal activity [16]. In European sea bass (*Dicentrarchus labrax*), H2B and H1 coding genes were cloned and their expression levels have been reported to be altered under stress conditions [14], and also after *Vibrio anguillarum* infection [17].

Nodavirus (NNV) is a naked bipartite single stranded RNA virus which severely affects European sea bass larvae and juveniles provoking high mortality rates [18, 19]. Nevertheless, other species such as the gilthead seabream (*Sparus aurata*) are infected without showing disease symptoms, acting as a natural reservoir for most of the virus strains [20]. NNV has demonstrated vertical transmission [21] and is able to colonize and replicate in very low levels into the European sea bass and gilthead seabream testis in order to not being detected by the immune response [22], altering the antimicrobial activities and pattern of expression of several AMPs [23].

In this study, we identify the complete sequences of H1 and H2B coding genes in European sea bass and gilthead seabream and study their pattern of expression in immune, reproductive and other important tissues in naïve specimens and under NNV infection. The results obtained, prompted us to analyse the modulation of both genes upon *in vitro* viral, bacterial infection, pathogen-associated molecular patterns (PAMPs) or mitogens stimulation of the immune response in gonad and/or head-kidney leucocytes (HKLs) in order to determine whether these two genes might have a role in the immune response of fish.

2. Material and methods

2.1. Animals

Healthy specimens of European sea bass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.) were bred and kept at the *Centro Oceanográfico de Murcia* (IEO, Mazarrón, Murcia) in 14 m^3 tanks with the water temperature ranging from 14.6 to 17.8 °C, flow-through circuit, suitable aeration, filtration systems and natural photoperiod. The environmental parameters, mortality and food intake, were recorded daily. Juvenile specimens of both species with a mean body weight (bw) of 325 ± 37.5 g were used for the analysis of constitutive gene expression in naïve conditions (see below). Adult specimens of both species with a bw of 774 ± 93 g were used for *in vitro* treatments of the gonads (see below). Juvenile specimens of European sea bass (n = 50) or gilthead seabream (n = 50) with a mean bw of 200 ± 15 g, were transported to the University of Murcia (Spain) aquaria in order to perform *in vivo* infections (see below). The experiments described comply with the Guidelines of the European Union Council (2010/63/UE). The protocol was approved by the Committee on the Ethics of Animal Experiments of the *Instituto Español de Oceanografía* (IEO)

(Permit Number: 2010/02) and of the University of Murcia (Permit Number: A13150104).

2.2. Fish sampling

All specimens were anesthetized with 40 µl/l of clove oil before sampling, then weighed, completely bled and immediately decapitated. Blood was obtained from the caudal peduncle and the serum samples, obtained by centrifugation (10,000 xg, 1 min, 4 °C), were immediately frozen in liquid nitrogen and stored at −80 °C until use.

In order to analyse the constitutive expression in naïve conditions, brain, gill, liver, skin, gonad, gut, head-kidney, spleen and thymus fragments from 6 independent fish were removed and immediately frozen in TRIzol® Reagent (Life Technologies) at -80 ºC until used for RNA isolation. HKL suspensions were obtained as previously described [24]. In brief, fragments of head-kidney tissue were transferred to 7 ml of sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35 % sodium chloride, 100 IU/ml penicillin (Life Technologies), 100 mg/ml streptomycin (Life Technologies) and 5 % fetal bovine serum (FBS; Life Technologies)] under sterile conditions. Cell suspensions were obtained by forcing fragments of the organ through a 100 µm nylon mesh, washed twice by centrifugation [400 xg, 10 min, room temperature (RT)], counted and adjusted to 10^7 cells/ml in sRPMI. In all cases, leucocyte viability was determined by the trypan blue exclusion test and resulted higher than 98 %.

2.3. Viruses and bacteria

NNV (strain 411/96, genotype RGNNV) was propagated in the SSN-1 cell line [19]. The SSN-1 cells were grown in Leibovitz's L-15 medium (Gibco) supplemented with 10 % FBS, 2 mM L-glutamine (Life Technologies), 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin (Gibco) at 25 °C using Falcon Primaria cell culture flasks (Becton Dickinson). Inoculated cells were incubated at 25 ºC until the cytopathic effect (CPE) was extensive. Supernatants were harvested and centrifuged to eliminate cell debris. Virus stock was titrated in 96-well plates and expressed as the viral dilution infecting 50 % of the cell cultures ($TCID_{50}$), following a methodology previously described [25].

Pathogenic bacteria *Vibrio anguillarum* (*Va*) R-82 and *Photobacterium damselae* subsp. piscicida (*Pd*) were grown in sTSB [tryptic soy broth (Laboratorios

Conda) supplemented with 1.5 % NaCl] at 22 °C for 24 h. Absorbance at 600 nm was measured and used to know the concentration based on growth curves. Both bacterial cell cultures were washed in sterile 0.01 M phosphate-buffered saline (PBS, pH 7.4) by centrifugation (6,000 xg, 15 min, 4 °C) and adjusted to 10^{10} bacteria/ml. For heat-killing, cultures were washed with PBS, incubated at 60 ºC for 30 min, washed and adjusted to 10^{10} bacteria/ml with 0.01 M PBS.

2.4. *In vivo* infection

Once at the University of Murcia (Spain) facilities, juvenile specimens (n= 50) of both species were randomly divided into two tanks, kept in 450–500 L running seawater (28 ‰ salinity) aquaria at 25 °C and with a 12 h light: 12 h dark photoperiod and acclimatised for 15 days prior to the infection. The infection was performed by intramuscular injection of 100 µl containing 10^6 $TCID_{50}$/fish of NNV in SSN-1 culture medium, a mock-infected group was injected with 100 µl of SSN-1 culture medium since this route of infection has been proven to be the most effective [26]. Fish (n = 5 fish/group and time) were sampled 1, 7 or 15 days upon infection and gonad and brain were removed and immediately frozen in TRIzol® Reagent and stored at -80 ºC for later RNA isolation as described below.

2.5. *In vitro* treatments

Fragments of European sea bass ovaries (n = 6) or testis (n = 6) or gilthead seabream gonads (n = 6) were removed, weighted and chopped into 1 mm^2 to culture them in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine, 100 IU./ml penicillin, 100 µg/ml streptomycin, 2 µg/ml fungizone (Life Technologies) and 2 % FBS. Six fragments of each tissue from independent fish specimens were incubated in flat-bottomed 96-well microtiter plates (Nunc) with 200 µl of: culture medium alone (control), NNV (10^7 $TCID_{50}$/ml), *Va* (4 x 10^7 bacteria/ml) or polyinosinic:polycytidic acid (pI:C; 62,5 µg/ml; Sigma) at 25 ºC during 24 h. Afterwards, the fragments of tissue were washed in 0.01 M PBS and stored in TRIzol® Reagent at -80 ºC for later isolation of RNA as described below.

HKLs from healthy fish (n = 5) were isolated and maintained in Leibovitz's L-15-medium supplemented with 10 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 20 mM HEPES (Gibco). Aliquots of 10^7 HKLs/ml were

incubated in flat-bottomed 48-well microtiter plates (Nunc) at 22 ºC during 24 h with: culture medium alone (control), 10^6 $TCID_{50}$ NNV/ml, 10^8 live bacteria/ml (*Va* or *Pd*), 50 µg/ml synthetic unmethylated cytosine-phosphodiester-guanosine oligodeoxynucleotide 1668 (CpG ODN 1668; sequence 5'-TCCATGACGTTCCTGATGCT-3'; Eurogentec), 25 µg/ml pI:C, 5 µg/ml lipopolysaccharide (LPS; Sigma), 10 µg/ml phytohemagglutinin (PHA; Sigma) or 5 µg/ml concanavalin A (ConA; Sigma). Afterwards, leucocytes were washed with 0.01M PBS and stored in TRIzol® Reagent at -80 ºC for later isolation of RNA as mentioned below.

2.6. Gene sequences search and bioinformatics analysis

Complete sequences of European sea bass *h1* and *h2b* genes were obtained from the European sea bass genome (http://seabass.mpipz.mpg.de/) and analysed for similarity with known orthologue sequences using the BLAST program [27] within the ExPASy Molecular Biology server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This program was also used to compare European sea bass sequences with the gilthead seabream expressed sequence tags (ESTs) databases. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [28] to confirm that they are *bona fide* gilthead seabream sequences.

The evolutionary history was inferred using the Neighbor-Joining method [29] and the optimal tree was obtained. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches [30]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [31] and are in the units of the number of amino acid substitutions per site.

2.7. Analysis of gene expression by real-time PCR

Total RNA was isolated from TRIzol® Reagent frozen samples following the manufacturer's instructions. One µg of total RNA was treated with DNAse I (Promega) to remove genomic DNA and the first strand of cDNA synthesized by reverse transcription using the Superscript III (Life Technologies) with an oligo-dT12-18 primer (Life Technologies) followed by RNAse H (Life Technologies) treatment. Real-

time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated at 95 ºC for 10 min, followed by 40 cycles of 15 s at 95 ºC, 1 min at 60 ºC , and finally 15 s at 95 ºC, 1 min at 60 ºC and 15 s at 95 ºC. For each mRNA, gene expression was corrected by the elongation factor 1 alpha (*ef1a*) expression in each sample and expressed as $2^{-\Delta Ct}$, where ΔCt is determined by subtracting the *ef1a* Ct value from the target Ct. The primers used, specific for the histone forms studied herein and described in section 2.6., were designed using the Oligo Perfect software tool (Thermo Fisher Scientific) and are shown in Table 1. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primer for specificity. All amplifications were performed in duplicate cDNAs and repeated once to confirm the results. Negative controls with no template were always included in the reactions.

2.8. Statistical analysis

Data were analysed by one-way ANOVA to denote statistical differences among groups, followed by Tukey's post-hoc tests, except in the in *vivo* experiment in which a t-Student test was used to determine statistical differences between infected and control groups. A non-parametric Kruskal–Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. Statistical analyses were conducted using SPSS 20 software. All data are presented as mean ± standard error of the mean (SEM). Minimum level of significance was fixed in 0.1.

3. Results

3.1 Identification of European sea bass and gilthead seabream *h1* and *h2b* gene sequences

Complete cDNA sequences coding for European sea bass proteins H1 and H2B were available at the European sea bass genome database (http://seabass.mpipz.mpg.de/). We found one uncharacterized clone containing the entire open reading frame (ORF) coding for each gilthead seabream proteins H1 and H2B at the EST databases available at the NCBI GenBank database [GenBank accession number *h1*: FM151953 (unpublished); *h2b*: AM953780 [32]]. The predicted

length, homology and e-values obtained from the gene sequences were compared with their human orthologues (Table 2) resulting in *bona fide* sequences.

Phylogenetic tree showed two distinct clades for H1 and H2B proteins (Fig. 1). The clustering provides evidences of high bootstrap support in the lineage of European sea bass and gilthead seabream. Moreover, the teleost H1 proteins form an exclusive clade opposed to human H1 sequences. Human H3 proteins were used as outgroup.

3.2. Expression of *h1* and *h2b* under naïve conditions

We found *h1* mRNA transcripts in brain, gills, liver, skin, gonad, gut, head-kidney, spleen, thymus and blood tissues from both species (Fig. 2), although some differences between species were observed. Thus, in European sea bass (Fig. 2a), the tissues with the highest expression of *h1* gene were in thymus and blood followed by brain and liver. Gills and gonad were the tissues with the lowest *h1* expression levels (10,000 fold lower than thymus). However, in the gilthead seabream (Fig. 2b), the blood showed the highest *h1* gene expression levels, followed by head-kidney and spleen whereas liver, gut and gonad showed the lowest expression (100,000-fold lower than blood).

Regarding the expression of H2B encoding gene (Fig. 3), no constitutive expression were observed in brain, skin and spleen of European sea bass (Fig. 3a), whilst the highest transcription levels were found in thymus. In contrast, in gilthead seabream (Fig. 3b), all tissues constitutively expressed this gene. The highest level of expression was found in gonad and blood while thymus has a medium level of expression and the lowest expression was observed in liver (10,000-fold lower than in blood). Overall, *h2b* transcription was lower than the expression of *h1* gene.

3.3. The expression of *h2b* but not of *h1* was increased in head-kidney upon NNV infection in both species

Transcription of both *h1* and *h2b* genes was significantly regulated by NNV infection (Fig. 4 and 5, supplementary table 1). The expression levels of *h1* were down-regulated in brain but up-regulated in testis of European sea bass after 7 days of NNV infection (Fig. 4a). In contrast, in gilthead seabream, the transcription levels of *h1* were down- and up-regulated in brain after 7 and 15 days post-infection, respectively, and down-regulated in gonad at day 15 post-infection (Fig. 4b).

In European sea bass (Fig. 5a), the *h2b* gene expression was down-regulated in brain at day 1 and in testis at day 15 post-infection whilst it was down-regulated in gilthead seabream gonad after 7 days (Fig. 5b). Interestingly, in both species the *h2b* transcription was up-regulated in head-kidney at different time post-infection (7 days in European sea bass or 15 days in gilthead seabream).

3.4. The expression of *h1* in European sea bass ovary and of *h2b* in the gilthead seabream testis were inhibited after some *in vitro* treatments

When we analysed the pattern of expression of *h1* in the gonad of European sea bass and gilthead seabream after 24 hours of *in vitro* treatment, we found that only European sea bass ovaries showed down-regulated *h1* gene expression levels after NNV infection, whilst in European sea bass testis and gilthead seabream gonad was unchanged (Fig. 6a). However, the pattern of expression of *h2b* gene in European sea bass gonads was unaltered by any treatment while was down-regulated after the challenge with *Va* and pI:C in gilthead seabream gonad (Fig. 6b).

3.5. The expression of *h2b* gene was exclusively up-regulated in gilthead seabream HKLs

Finally, we studied the pattern of expression of *h1* (Fig. 7) and *h2b* (Fig. 8) genes in HKLs after 24 hours of treatment with known immune *stimuli* and our data showed that *h1* gene expression was down-regulated after NNV, *Va* or *Pd* treatment in European sea bass HKLs (Fig. 7a), and after NNV, *Va*, LPS, PHA or ConA treatment in gilthead seabream HKLs (Fig. 7b).

Similarly, the *h2b* gene expression in HKLs of European sea bass was down-regulated upon NNV, *Va*, *Pd*, pI:C or ConA treatments (Fig. 8a). In contrast to this and what happened with *h1* gene expression, in gilthead seabream HKLs the *h2b* gene expression was up-regulated after all immune *stimuli* assayed except with PHA (Fig. 8b).

4. Discussion

Histones, as chromatin structure proteins, were thought to be confined to the nucleus. However, different studies have detected various histones and their fragments in the cytoplasm of several cell types including leucocytes from mammals, bird, frogs,

295 fish and shrimps, showing those proteins a broad spectrum of antimicrobial activities
296 [33]. In fact, upon immune stimulation, leucocyte histones, mainly from macrophages
297 and neutrophils, are mobilized from the nucleus to the cytoplasm, the membrane and
298 even secreted to form the extracellular NETs [33] but no information exists at gene
299 level. In mammals, regarding to this, histones not only appear on the surface of
300 apoptotic cells but also on viable cell such as T-lymphocytes, macrophages or intestine
301 epithelial cells [33], whilst in fish they have been described on the cell surface of
302 macrophages, natural cytotoxic cells (NCC) [34, 35] and in the mucosa of gill and skin
303 tissues [6, 36]. In addition, histones are demonstrated to be innate immune effectors in a
304 wide range of tissues, being involved in the interaction with pathogens showing both
305 lytic activity and helping in their internalization through endocytic vesicles [33].
306 Although the mechanism of action of these histones is not completely known yet, a
307 specific conformation of histones and histone fragments is needed, suggesting that their
308 immune function is not only a consequence of their high amount of basic residues [37].

309 Both, core (H2A, H2B) and the linker (H1) histones showed antimicrobial
310 activity in several fish species [4, 6, 11, 13, 15, 33]. Concretely, in the European sea
311 bass, partial cDNA sequences coding for H1 and H2B proteins were isolated and their
312 pattern of expression analysed under stress conditions, resulted on a similar pattern of
313 expression in gills and epidermis than haemoglobin-like protein [14], a known
314 antimicrobial peptide [38]. In the present work we used the complete sequences coding
315 for these proteins for searching the gilthead seabream orthologue sequences. Thus, we
316 found two sequences annotated but not characterized in the GenBank database. Though
317 several histone forms are probably present in seabream and sea bass we only focused on
318 those previously documented [14, 32], which in addition showed good relation with
319 their zebrafish and human orthologues as evidenced by the phylogenetic tree,
320 suggesting that their function could be also conserved.

321 Since histones with antimicrobial activity were firstly identified in fish skin,
322 most studies in fish have avoided the study of the constitutive expression of histones in
323 other tissues apart from skin or liver [4, 8, 13], but other AMPs have been localized in a
324 wide range of tissues including immune-privileged tissues as brain or gonads [15, 23].
325 Our data showed that *h1* was constitutively expressed in all the tissues analysed in both,
326 European sea bass and gilthead seabream, as also occurred in the olive flounder [16].
327 Interestingly, in the olive flounder the highest expression of *h1* gene was found in gonad

[16], however, our data showed the highest *h1* gene expression in immune tissues of both species. As far as we are concern, our study is the first analysing the pattern of expression of *h2b* gene covering most of the tissues in fish. Therefore, we found that in European sea bass, *h2b* gene transcription was not detected in brain, skin or spleen but, was highly expressed in thymus. In contrast, in gilthead seabream *h2b* gene was highly expressed in peripheral blood and gonad.

We next analysed the pattern of expression of *h1* and *h2b* upon NNV infection, a virus which target tissues are the retina and brain [39] and colonizes the gonad to be vertically transmitted [22]. Our data showed that upon *in vivo* infections with NNV, *h1* is up-regulated in the gonad of European sea bass and in the brain of gilthead seabream, which is greatly correlated to the increased immunity in seabream brain and sea bass gonad as determined by the transcription levels of interferon, AMPs and leucocyte markers [22, 23, 40], while *h2b* is up-regulated in the head-kidney of both species. However, whether this is related to inflammation, immune response or tissue damage or reparation merits further investigation. Interestingly, in Rohu (*Labeo rohita*), LHH1M protein, that corresponds with the linker histone H1, is up-regulated in the brain of specimens resistant to gram negative bacteria *Aeromonas hydrophila* [41] as occurred with gilthead seabream, which is an asymptomatic carrier species of the NNV strain used to perform the experiment [20]. On the other hand, European sea bass is very susceptible to NNV [18] and our data showed that *h1* expression was down-regulated in the virus target tissue, the brain, which is suffering great damage and no reparation is performed. These data could suggest that histones are mobilized from the nucleus to other cellular locations and this might lead to the down-regulation of histone genes.

Taking into account the high expression of *h2b* gene in the gonad of gilthead seabream, the immune-privileged status of the gonad [42, 43] and the ability of NNV to colonize the testis [22], we have analysed the expression of *h1* and *h2b* genes in mature European sea bass male and females and gilthead seabream male gonads upon *in vitro* treatment with alive NNV or *Va* or pI:C, and found slight down-regulations of *h1* expression in the European sea bass ovary upon NNV infection and of *h2b* expression in the gilthead seabream gonad upon *Va* and pI:C treatment. These data suggest that the transcriptional changes observed on both genes upon *in vivo* infections were orchestrated by the systemic immune response. However, it has been recently demonstrated the presence of NNV in the testis of both species upon an infection [22].

Probably, the existence of other specific AMPs in the gonad together with the high proliferative rates that this tissue showed during gametogenesis, avoids the use of H1 and H2B as antimicrobial proteins, whilst in other tissue such as HKLs or brain, this function is enhanced and needed.

Histones are well known to be shed out of the cells in mammalian neutrophils extracellular traps (NETs) and recently these NETs have been described to be produced by some leucocytes of mainly cyprinid fish species [44-47]. In that sense and taking into account the high expression of *h1* and of *h1* and *h2b* observed in European sea bass and gilthead seabream blood, respectively, we next analysed the transcription levels of these genes in HKLs stimulated with different immune stimuli. Thus, we observed that *h1* gene expression was down-regulated in European sea bass upon challenge with live virus and bacteria, while in gilthead seabream this down-regulation was also observed upon LPS, PHA and ConA treatments. In human monocytes, and upon LPS stimulation, H1 is able to bind LPS [48]. In contrast to what happened to *h1* expression, the transcription of *h2b* gene was up-regulated in gilthead seabream HKLs and down-regulated in European sea bass HKLs upon NNV and other immune *stimuli*. Although further studies are needed, this study clearly suggests that the ability to use histones as AMPs, either in traps or not, might be a clear difference in the susceptibility to infections of each fish species.

5. Conclusions

In conclusion, this is the first study analysing the pattern of expression of H1 and H2B coding sequences in a broad spectrum of tissues of European sea bass and gilthead seabream fish species. Moreover, both genes are regulated in different tissues by pathogens, PAMPs and mitogens pointing to an important role in fish immunity. Thus, our data suggest that H1 might have a role in the immune response against NNV in the brain of both species, due to the fact that *h1* expression pattern is similar to that found for other AMPs and several IFN pathway genes and correlated well with the different susceptibility to infection of both species [23, 40]. In the other hand, *h2b* expression seems to be more important in the head-kidney and HKLs immune response. Nevertheless, further functional studies are needed to understand histones implication in fish immunity, and concretely in antimicrobial responses upon NNV infection, and several studies are in progress in our laboratory.

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Appendix A. Suplementary data

Supplementary table 1: Mean values of *h1* and *h2b* transcription in European sea bass and gilthead seabream brain, gonad and head-kidney tissues after 1, 7 and 15 days of infection with NNV. Data were corrected with *ef1a* gene expression levels and normalized with the mean of control group (mock-infected). Asterisks denote statistical differences (t Student test; *P<0.1, **P < 0.05) with control group.

	***h1* gene expression**		
	days	European sea bass	Gilthead seabream
Brain	1	0.55±0.19	0.63±0.24
	7	0.12±0.02*	0.39±0.10*
	15	0.95±0.34	2.75±0.64**
Gonad	1	1.10±0.34	0.84±0.40
	7	2.28±0.92*	0.26±0.10
	15	1.06±0.39	0.32±0.11*
Head-kidney	1	0.04±0.03	1.04±0.20
	7	0.38±0.16	0.43±0.10
	15	0.34±0.14	1.69±1.10

	***h2b* gene expression**		
	days	European sea bass	Gilthead seabream
Brain	1	0.14±0.03**	0.94±0.43
	7	0.30±0.07	0.83±0.29
	15	2.44±0.75	1.34±0.30
Gonad	1	1.27±0.67	0.95±0.34
	7	1.22±0.43	0.53±0.19 *
	15	0.21±0.11**	0.65±0.24
Head-kidney	1	0.16±0.07	1.15±0.27
	7	4.57±1.88**	0.72±0.27
	15	3.31±1.86	2.72±0.93*

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544

545 **Figure 1:** Phylogenetic analysis of the H1 and H2B proteins of European sea bass and
546 gilthead seabream with related sequences of fish and mammalian histone proteins. The
547 phylogenetic tree was drawn following the Neighbor-Joining method for the analysis of
548 evolutionary relationship. Genbank accession numbers are shown in parentheses.
549 Histones with "t" are thymus isolated histones.

550

551 **Figure 2:** Levels of expression of *h1* gene in European sea bass (a) and gilthead
552 seabream (b) tissues: brain (Br), gills (Gi), liver (Li), skin (Sk), gonad (Go), gut, head-
553 kidney (Hk), spleen (Sp), thymus (Th) and blood (Blo) studied by real-time PCR. Data
554 represent mean relative expression to the expression of endogenous control *ef1a* gene ±
555 SEM (n = 6). Letters denote statistical differences among tissues according to ANOVA
556 and Tukey's post-hoc test ($P < 0.05$).

557

558 **Figure 3:** Levels of expression of *h2b* gene in European sea bass (a) and gilthead
559 seabream (b) tissues: brain (Br), gills (Gi), liver (Li), skin (Sk), gonad (Go), gut, head-
560 kidney (Hk), spleen (Sp), thymus (Th) and blood (Blo) studied by real-time PCR. Data
561 represent mean relative expression to the expression of endogenous control *ef1a* gene ±
562 SEM (n = 6). Letters denote statistical differences among tissues according to ANOVA
563 and Tukey's post-hoc test ($P < 0.05$). ND, non detected.

564

565 **Figure 4:** Expression levels of *h1* gene in European sea bass (a) and gilthead seabream
566 (b) brain, gonad and head-kidney after 1, 7 and 15 days of *in vivo* NNV infection (10^6
567 $TCID_{50}$ per fish) studied by real-time PCR. Data are expressed as the mean ± SEM (n =
568 5) of mRNA fold increase respect to control samples. Asterisks denote significant
569 differences with the controls at each sampling time (t Student test; *$P<0.1$, **$P < 0.05$).

570

571 **Figure 5:** Expression levels of *h2b* gene in European sea bass (a) and gilthead seabream
572 (b) brain, gonad and head-kidney after 1, 7 and 15 days of *in vivo* NNV infection (10^6
573 $TCID_{50}$ per fish) studied by real-time PCR. Data are expressed as the mean ± SEM (n =
574 5) of mRNA fold increase respect to control samples. Asterisk denote significant

differences with controls at each sampling time (t Student test; *P<0.1, **P < 0.05).

Figure 6: Expression levels of *h1* (a) and *h2b* (b) genes in European sea bass testis and ovaries and gilthead seabream gonad after 24 h of *in vitro* challenge with NNV (10^7 $TCID_{50}$/ml), *Va* (4×10^7 bacteria/ml) and poly I:C (pI:C 62,5 µg/ml) studied by real-time PCR. Data are expressed as the mean ± SEM (n = 6) of mRNA transcripts relative to *ef1a* gene expression. Letters denote statistical differences among tissues according to ANOVA and Tukey's post-hoc test (P < 0.05).

Figure 7: The expression of *h1* gene in HKLs of European sea bass (a) and gilthead seabream (b) after 24 h of *in vitro* challenge with culture medium alone (control), 10^6 $TCID_{50}$ NNV/ml, 10^8 live bacteria/ml (*Va* or *Pd*), 50 µg/ml CpG ODN 1668, 25 µg/ml pI:C, 5 µg/ml LPS, 10 µg/ml PHA or 5 µg/ml ConA studied by real-time PCR. Data are expressed as the mean ± SEM (n = 5) of mRNA transcripts relative to *ef1a* gene expression.). Letters denote statistical differences among tissues according to ANOVA and Tukey's post-hoc test (P < 0.05).

Figure 8: The expression of *h2b* gene in HKLs of European sea bass (a) or gilthead seabream (b) after 24 h of *in vitro* challenge with culture medium alone (control), 10^6 $TCID_{50}$ NNV/ml, 10^8 live bacteria/ml (*Va* or *Pd*), 50 µg/ml CpG ODN 1668, 25 µg/ml pI:C, 5 µg/ml LPS, 10 µg/ml PHA or 5 µg/ml ConA studied by real-time PCR. Data are expressed as the mean ± SEM (n = 5) of mRNA transcripts relative to *ef1a* gene expression.). Letters denote statistical differences among tissues according to ANOVA and Tukey's post-hoc test (P < 0.05).

Table 1: Primers used for analysis of gene expression by real-time PCR.

Species	Molecule	Gene Abbrev.	Accession number	Primer sequence
European sea bass	Histone 1	*h1*	DLAgn00119260	AAGAAGACGGGTCCCTCAGT
				CTTGACCTTCTTCGCTTTGG
	Histone 2B	*h2b*	DLAgn00179560	GGAGAGCTACGCCATCTACG
				GCTCAAAGATGTCGCTCACA
	Elongation factor 1 alpha	*ef1a*	AJ866727	CGTTGGCTTCAACATCAAGA
				GAAGTTGTCTGCTCCCTTGG
Gilthead seabream	Histone 1	*h1*	FM151953	CGTGGTGAAGAACAGAGCAA
				TTGACCCTTTTCGTCTTTGG
	Histone 2B	*h2b*	AM953480	AGACGGTCAAAGCACCAAAG
				AGTTCATGATGCCCATAGCC
	Elongation factor 1 alpha	*ef1a*	AF184170	CTGTCAAGGAAATCCGTCGT
				TGACCTGAGCGTTGAAGTTG

605 **Table 2:** Identity (in %; [a]) and e-value ([b]) of the predicted proteins respect to the human
606 orthologues. Asterisk denotes the sequences with predicted full length.

607

Predicted protein	Fish species	Gene accession number	Protein length	Identity[a]	e-value[b]
H1	Sea bass	DLAgn_0011926	188*	71	1e-25
	Seabream	FM151953	192*	67	9e-23
	Zebrafish	XP_017209709	199*	63	1e-31
	Human	NP_005313	226*		
H2B	Sea bass	DLAgn_00179560	121*	92	6e-67
	Seabream	AM953480	134*	95	5e-67
	Zebrafish	NP_001013481	124*	98	2e-72
	Human	AAH98112	124*		

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Figure 1

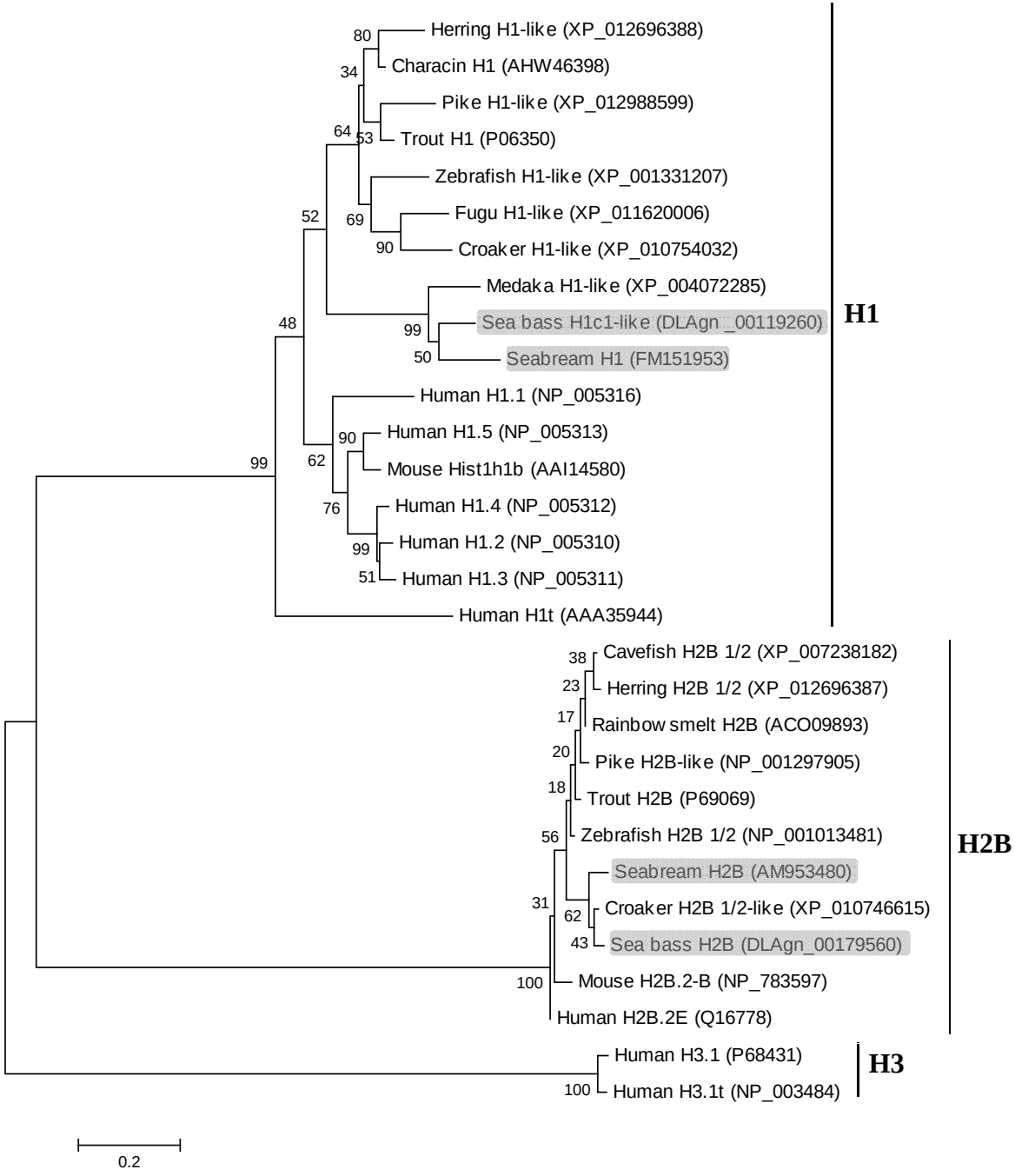
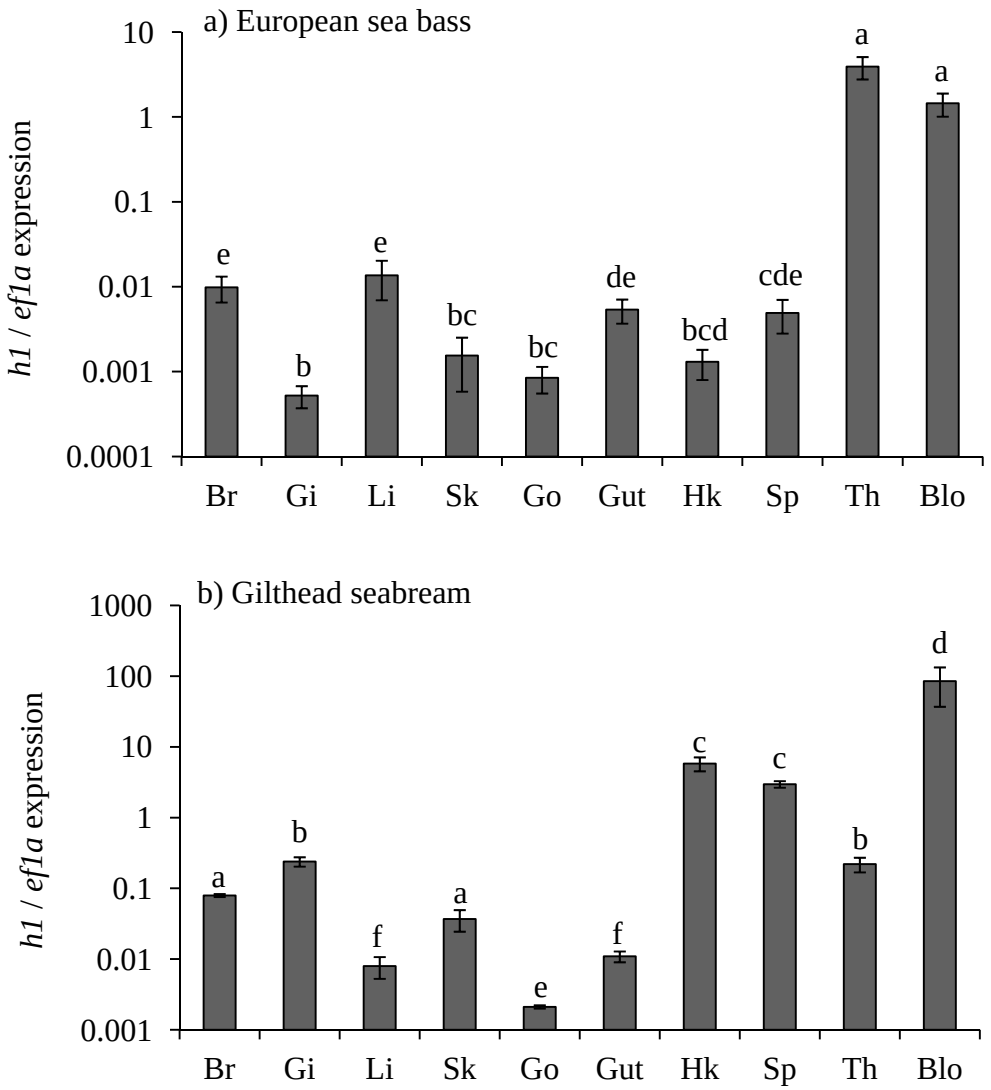

Herring H1-like (XP_012696388)
Characin H1 (AHW46398)
Pike H1-like (XP_012988599)
Trout H1 (P06350)
Zebrafish H1-like (XP_001331207)
Fugu H1-like (XP_011620006)
Croaker H1-like (XP_010754032)
Medaka H1-like (XP_004072285)
Sea bass H1c1-like (DLAgn _00119260)
Seabream H1 (FM151953)
Human H1.1 (NP_005316)
Human H1.5 (NP_005313)
Mouse Hist1h1b (AAI14580)
Human H1.4 (NP_005312)
Human H1.2 (NP_005310)
Human H1.3 (NP_005311)
Human H1t (AAA35944)
H1
Cavefish H2B 1/2 (XP_007238182)
Herring H2B 1/2 (XP_012696387)
Rainbow smelt H2B (ACO09893)
Pike H2B-like (NP_001297905)
Trout H2B (P69069)
Zebrafish H2B 1/2 (NP_001013481)
Seabream H2B (AM953480)
Croaker H2B 1/2-like (XP_010746615)
Sea bass H2B (DLAgn_00179560)
Mouse H2B.2-B (NP_783597)
Human H2B.2E (Q16778)
H2B
Human H3.1 (P68431)
Human H3.1t (NP_003484)
H3
0.2

Figure 2


a) European sea bass
h1 / ef1a expression
10
1
0.1
0.01
0.001
0.0001
e
b
e
bc
bc
de
bcd
cde
a
a
Br
Gi
Li
Sk
Go
Gut
Hk
Sp
Th
Blo
b) Gilthead seabream
h1 / ef1a expression
1000
100
10
1
0.1
0.01
0.001
a
b
f
a
e
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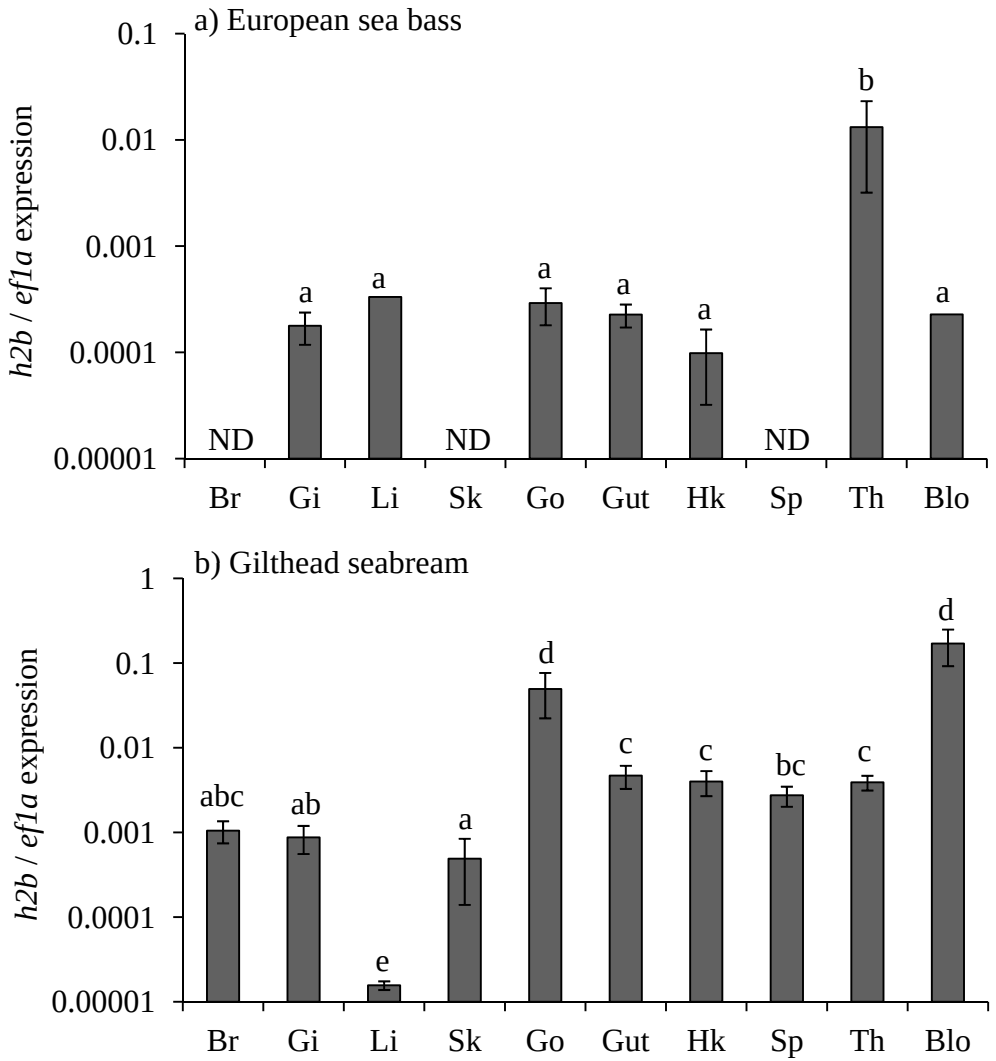
c
c
b
d
Br
Gi
Li
Sk
Go
Gut
Hk
Sp
Th
Blo

Figure 3


a) European sea bass
h2b / ef1a expression
0.1
0.01
0.001
0.0001
0.00001
ND
a
a
ND
a
a
a
ND
b
a
Br
Gi
Li
Sk
Go
Gut
Hk
Sp
Th
Blo
b) Gilthead seabream
h2b / ef1a expression
1
0.1
0.01
0.001
0.0001
0.00001
abc
ab
e
a
d
c
c
bc
c
d
Br
Gi
Li
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Hk
Sp
Th
Blo

Figure 4

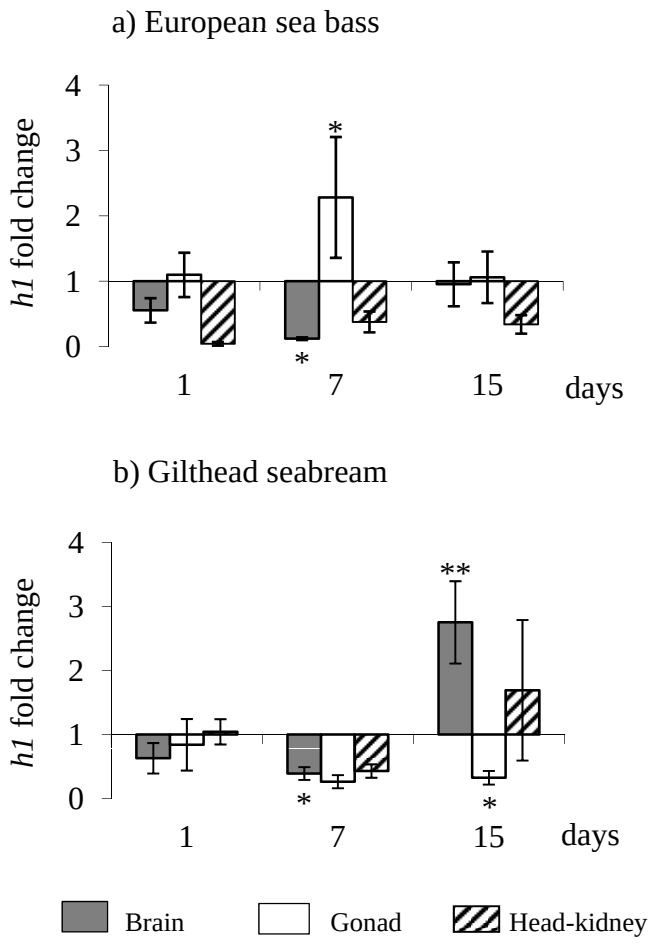

a) European sea bass
h1 fold change
4
3
2
1
0
*
*
1
7
15
days
b) Gilthead seabream
h1 fold change
4
3
2
1
0
**
*
*
1
7
15
days
Brain
Gonad
Head-kidney

Figure 5

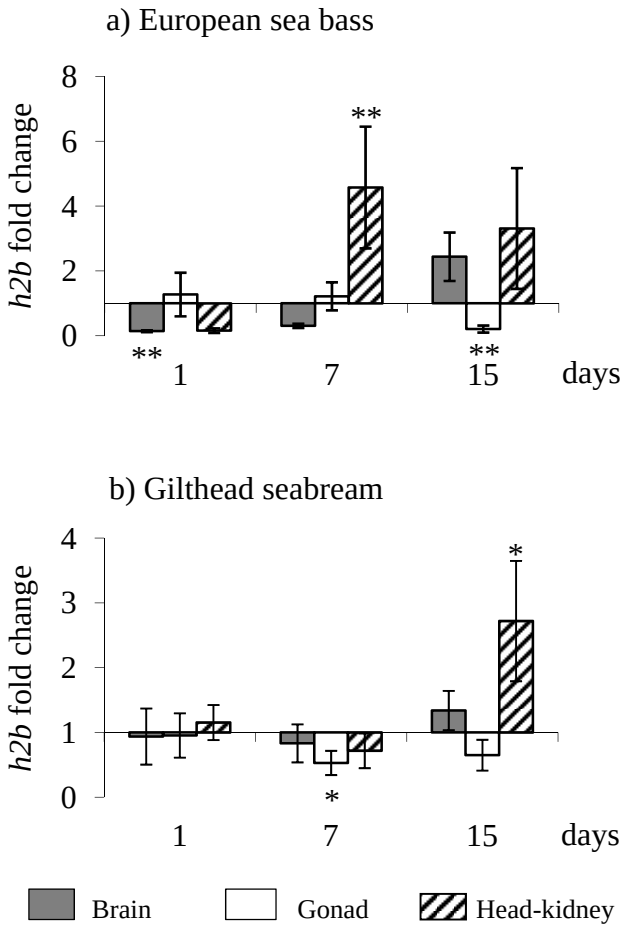

a) European sea bass
h2b fold change
8
6
4
2
0
**
**
**
1
7
15
days
b) Gilthead seabream
h2b fold change
4
3
2
1
0
*
*
1
7
15
days
Brain
Gonad
Head-kidney

Figure 6

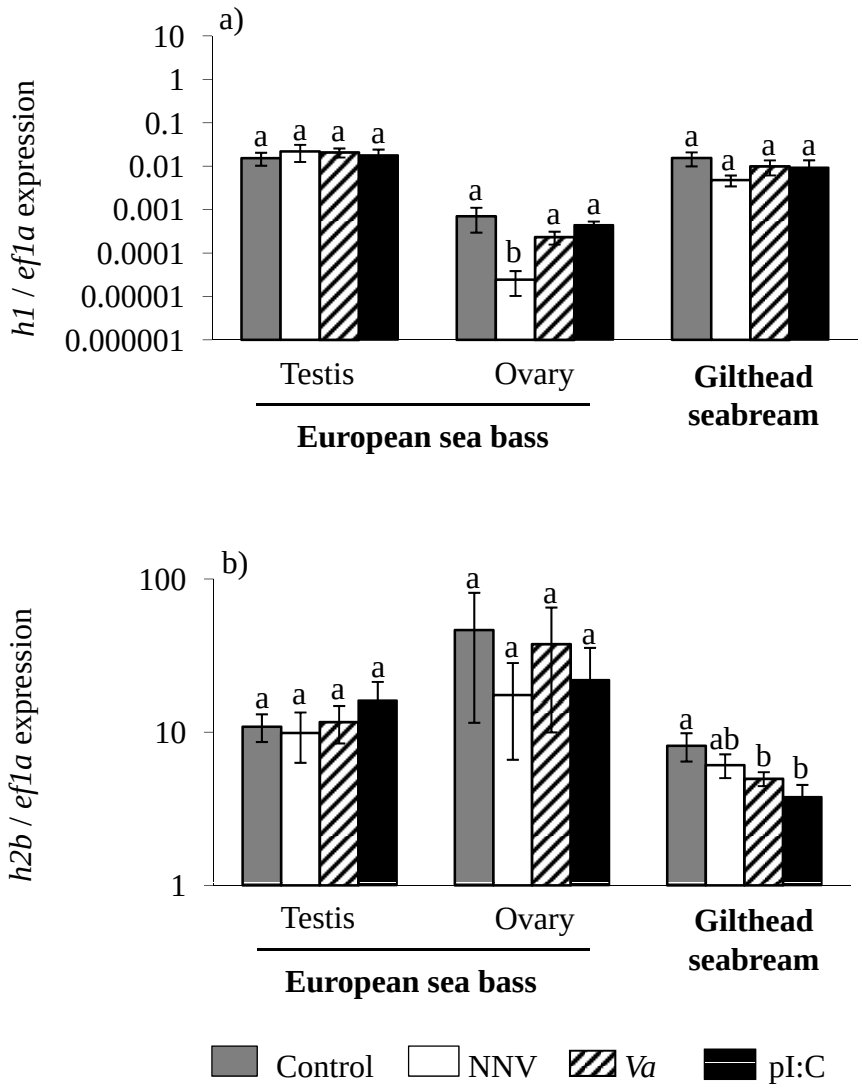

a)
h1 / ef1a expression
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
a a a a
a b a a
a a a a
Testis
Ovary
Gilthead seabream
European sea bass
b)
h2b / ef1a expression
100
10
1
a a a a
a a a a
a ab b b
Testis
Ovary
Gilthead seabream
European sea bass
Control
NNV
Va
pI:C

Figure 7

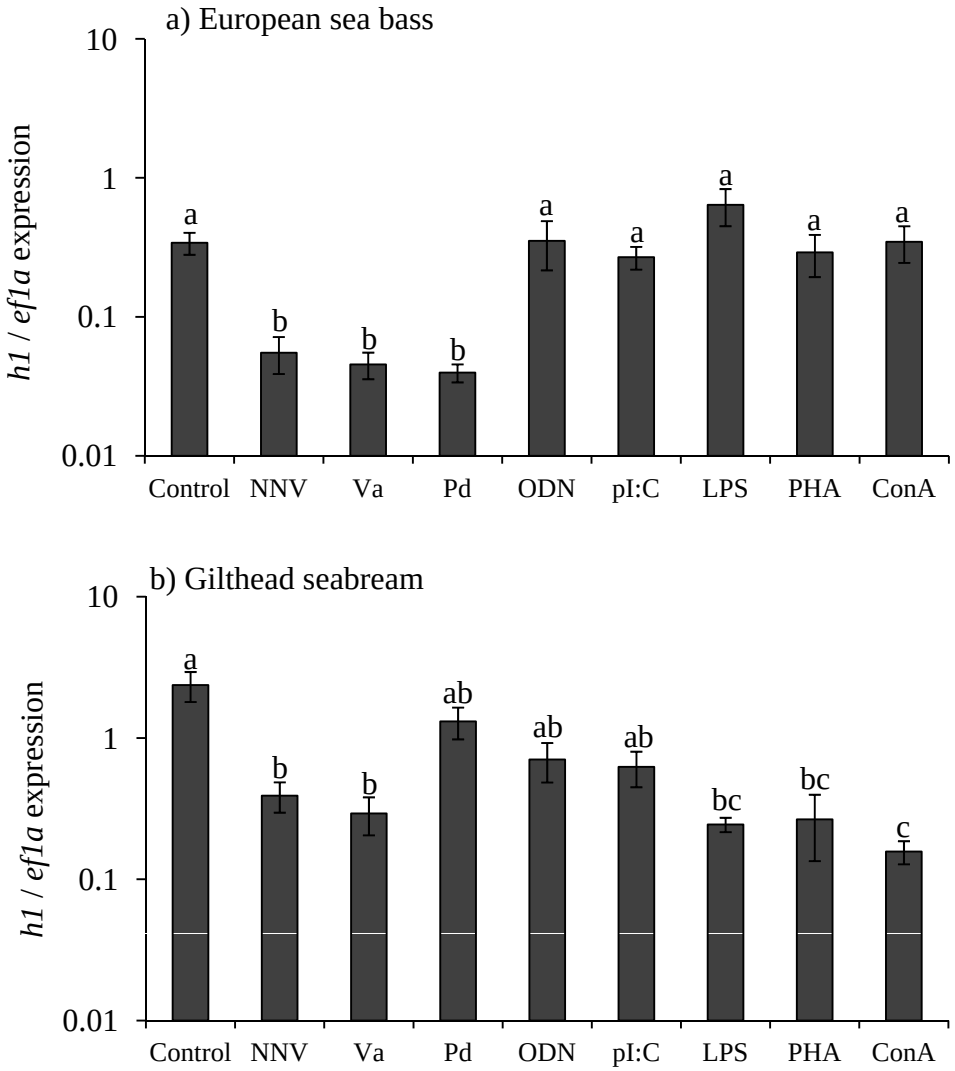
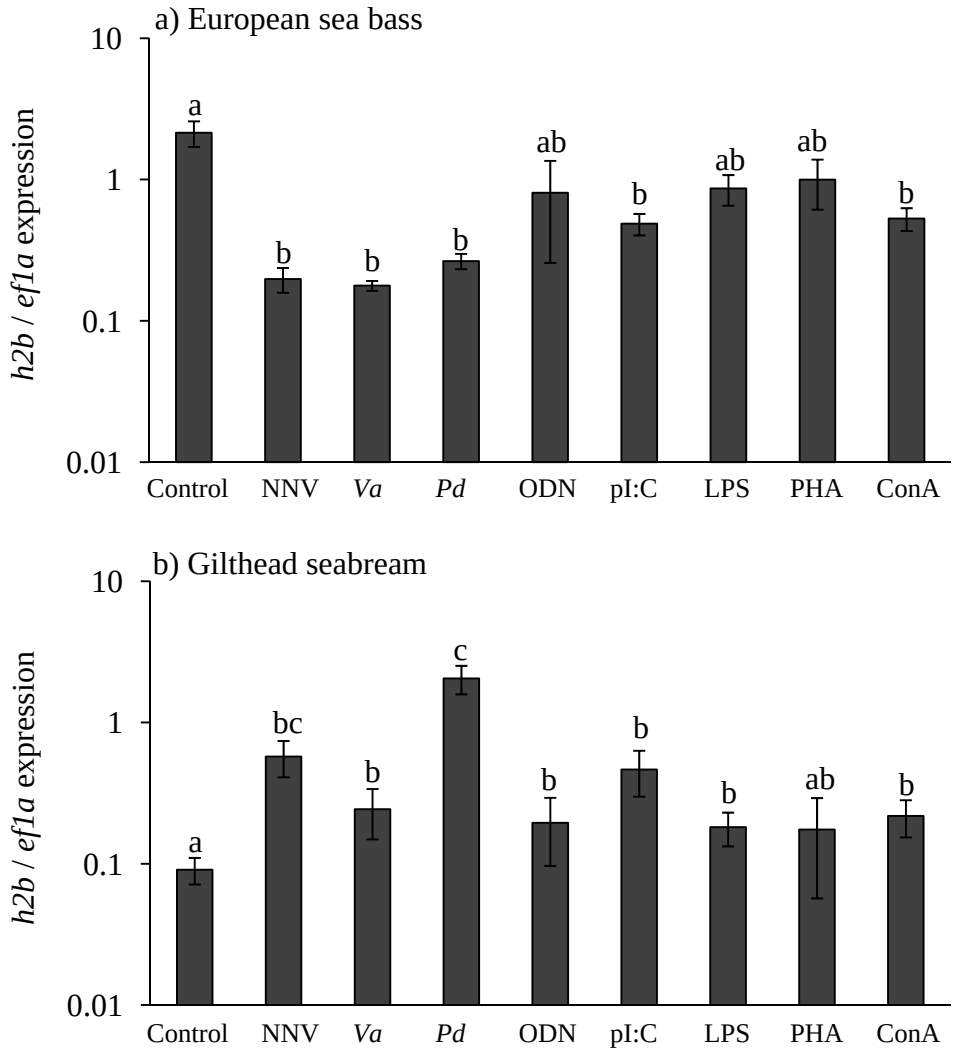

a) European sea bass
10
1
0.1
0.01
h1 / ef1a expression
a
b
b
b
a
a
a
a
a
Control
NNV
Va
Pd
ODN
pI:C
LPS
PHA
ConA
b) Gilthead seabream
10
1
0.1
0.01
h1 / ef1a expression
a
b
b
ab
ab
ab
bc
bc
c
Control
NNV
Va
Pd
ODN
pI:C
LPS
PHA
ConA

Figure 8


a) European sea bass
10
1
0.1
0.01
h2b / ef1a expression
a
b
b
b
ab
b
ab
ab
b
Control
NNV
Va
Pd
ODN
pI:C
LPS
PHA
ConA
b) Gilthead seabream
10
1
0.1
0.01
h2b / ef1a expression
a
bc
b
c
b
b
b
ab
b
Control
NNV
Va
Pd
ODN
pI:C
LPS
PHA
ConA

Highlights

- Histones H1 and H2b are characterized in European sea bass and gilthead seabream
- The transcription of *h1* gene may be related with immune response against NNV
- The transcription of *h2b* gene may be relevant in HKLs immune response against NNV
- The transcription of *h1* and *h2b* are differently regulated in HKLs